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Comparative transcriptome analysis of the hepatopancreas of Eriocheir sinensis following oral gavage with enrofloxacin

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1 Comparative transcriptome analysis of the hepatopancreas of *Eriocheir*
2 *sinensis* following oral gavage with enrofloxacin

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Abstract

Enrofloxacin is an important drug that is widely used in the treatment of the diseased *E. sinensis*. This study compared transcriptome differences in the hepatopancreas of *E. sinensis* following oral gavage with enrofloxacin. Our study produced 80,228,728 and 88,888,706 raw reads from control (kongbai3) and treatment (shiyuan4) groups, and after filtering and quality checks of the raw sequence reads, our analysis yielded 78,843,613 and 87,628,922 clean reads with an average length of 126bp from control and treatment groups, respectively. A total of 15,797 transcripts were assembled, with 11,975 transcripts were annotated. Moreover, 2,795 transcripts were judged to be differentially expressed genes. GO terms biological process and metabolic process were the most enriched in the oxidation-reduction process, translational initiation, membrane, cytoplasmic part and hydrolase activity. KEGG pathway analysis showed that metabolic and signal transduction pathways were significantly enriched. Furthermore, we found that *gshB* and the *CYP450* enzyme system plays a role in the metabolism of enrofloxacin in the hepatopancreas of *E. sinensis*. This study identified differential transcripts related to transmembrane transport and drug metabolism in *E. sinensis* which could help us developing our understanding of the molecular basis of enrofloxacin metabolism in this economically important aquaculture species.

Key words: *Eriocheir sinensis*; enrofloxacin; hepatopancreas; transcriptome;

38 Introduction

39 The Chinese mitten crab (*Eriocheir sinensis*) is one of the most important
40 aquaculture species in China, and its culture under facility conditions began in the
41 early 1980's (Li et al. 2007). Although commercial production is rapidly expanding,
42 this industry has been impeded by outbreaks of significant infectious diseases,
43 resulting in serious economic consequences. Causative organisms of diseases in *E.*
44 *sinensis* include viruses, bacteria, fungi and parasites (Chen et al. 2007; Bonami et al.
45 2011; Mu et al. 2011). At present, specific drugs for the treatment of disease in *E.*
46 *sinensis* is absent and most existing formulations are human or veterinary drugs. The
47 use of antimicrobials to reduce such problems has become an important aspect of crab
48 culture. Because of the special type of injection needed, and the difficult sampling
49 procedure involved, research on the kinetic behavior of such antimicrobials is limited
50 (Wu et al. 2006). Since there is no reasonable drug standard, the ill-informed use of
51 these antimicrobials could lead to the phenomenon of drug abuse, which not only
52 leads to the generation of drug resistance in the target population, but also bears
53 potential safety problems. To avoid drug abuse and ecological pollution, the most
54 important outstanding task is to investigate the metabolism of enrofloxacin and
55 identify appropriate drugs with which to prevent and control diseases in *E. sinensis*.

56 Enrofloxacin is an antimicrobial agent belonging to the third generation of
57 fluoroquinolone antimicrobials. Due to its broad antibacterial spectrum and high
58 potency, enrofloxacin is commonly used to treat bacterial infections afflicting crab
59 farming in China (Martinez et al. 2005; Tang et al. 2006). Enrofloxacin is bactericidal,

60 acting by inhibiting the DNA gyrase enzyme. This agent is biotransformed in the body
61 by N-dealkylation into a pharmacologically-active metabolite, ciprofloxacin (Rao et al.
62 2002). After oral administration, enrofloxacin is well absorbed, distributed into tissues,
63 and mainly excreted by the urine and feces (Intorre et al. 2000). Thus far, only the
64 general pharmacokinetics of enrofloxacin have been studied in *E. sinensis*. Following
65 the intramuscular injection of enrofloxacin, the drug residue reached maximal levels
66 in the hepatopancreas. Comparative pharmacokinetics showed fast absorption, broad
67 distribution and fast elimination of enrofloxacin in *E. sinensis* after intramuscular
68 dosing (Tang et al. 2006). The distribution of enrofloxacin in tissue is also influenced
69 by different salinities (Fang et al. 2007). However, the metabolic pathway of
70 enrofloxacin in *E. sinensis* body has not been elucidated. Recently, high-throughput
71 RNA sequence (RNA-seq) technology has provided a powerful and efficient method
72 for transcript analysis and metabolic gene discovery.

73 In crustaceans, it is an important immune organ for the hepatopancreas and the
74 primary site for the synthesis and excretion of immune molecules, such as
75 beta-1,3-glucan binding protein (LGBP) (Roux et al. 2002), antibacterial peptide
76 (AMP), lectin or lectin related proteins and others (Ried et al. 1996). Expressed
77 sequence tag (EST) analysis and gene discovery studies of *Litopenaeus vannamei* and
78 *Litopenaeus setiferus* also demonstrated that the hepatopancreas played a crucial role
79 in innate immunity and that a hepatopancreas cDNA library appeared to be more
80 diverse than a library synthesized from hemocytes (Gross et al. 2001). Previously,
81 Xihong Li et al (Li et al. 2013) constructed a non-normalized hepatopancreas cDNA

library from *E. sinensis* by high-throughput RNA sequencing. In the present study, we analyzed the hepatopancreas transcriptome of *E. sinensis* following oral gavage with enrofloxacin. This was useful in order to understand the biological function of the hepatopancreas and lay the foundation for further, more in-depth investigations of the Chinese mitten crab (*E. sinensis*). Characterizing immune molecules, understanding defense mechanisms, and comprehending drug metabolism, are critical in maintaining a healthy crab population in aquaculture, and avoiding the inappropriate use of drugs to combat disease.

Materials and Methods

Maintenance and treatment of Eriocheir sinensis

Experimental crabs were caught by fisherman from a commercial crab farm near Dongtai City, Jiangsu Province, China between October and December in 2014. In total, thirty male mitten crabs were further selected and maintained in a 40 m² concrete tank (Length×Width×Depth=5m×8m×1m). Ten healthy, sexually mature, male mitten crabs (*Eriocheir sinensis*, weighing 100 to 120g) that had reached the stage of rapid development were selected to be laboratory animals. These individuals were cultured in glass tanks with adequate aeration, temperature (24°C). Five crabs were orally treated with enrofloxacin (10mg/kg, Shanghai Guoyao Chemical Reagent Co. Shanghai, China) in the treatment groups (shiyang4). An additional, five crabs were orally treated with sterile H₂O as a control in the control groups (kongbai3). Based on our previous observations, T_{max} (the time to reach the highest concentration of the drug in the body) takes approximately one hour for the hepatopancreas of *E.*

104 *sinensis*. One hour later, the crabs were dissected on ice, and the hepatopancreas
105 removed from each crab was immediately placed in liquid nitrogen and stored at
106 -80°C for subsequence analysis.

107 *RNA isolation, RNA sequencing (RNA-seq) library construction, and sequencing*

108 Each frozen sample was ground in a mortar with liquid nitrogen, and total RNA
109 was extracted from approximately 80mg of hepatopancreas tissue with TRIzol reagent
110 (Invitrogen, USA) in accordance with the manufacturer's instructions. DNA
111 contaminants were removed after treatment with RNase-free DNase I (Takara
112 Biotechnology, Dalian, China). The final total RNA was dissolved in 200µL
113 RNase-free water. The concentration of total RNA was determined using a
114 Nano-Drop2000 spectrophotometer (Thermo Scientific, USA), and RNA integrity
115 was checked using an RNA 6000 Pico LabChip with the Agilent 2100 bioanalyzer
116 (Agilent, USA). Total RNA was incubated with 10 U DNase I (Ambion, USA) at
117 37°C for 1h, and then nuclease-free water was added to dilute the sample volume to
118 250µL. The messenger RNA (mRNA) was further purified with a MicroPoly(A)
119 Purist Kit (Ambion, USA) in accordance with the manufacturer's protocol. The
120 mRNA was dissolved in 100µL of RNA Storage Solution (Ambion), and then purified
121 using oligo-dT magnetic beads and fragmented by treating with divalent cations and
122 heat, followed by reverse transcription into cDNA using reverse transcriptase and
123 random hexamer-primers. This was followed by second strand cDNA synthesis using
124 DNA polymerase I and RNaseH. The resultant double-stranded cDNA was
125 end-repaired using T4 DNA polymerase, Klenow fragment, and T4 polynucleotide

126 kinase followed by a single (A) base addition using Klenow 3' to 5' exo-polymerase.
127 This was then ligated with an adaptor or index adaptor using T4 quick DNA ligase.
128 The size range of adaptor-modified fragments was selected by gel purification and
129 subject to PCR amplification as templates. After validation with an Agilent 2100
130 Bioanalyzer and ABI StepOnePlus Real-time PCR system, the cDNA library was
131 sequenced on a flow cell using the Illumina HiSeq 2500 (Illumina, SanDiego, USA).

132 *Sequencing, data processing and quality control*

133 We filtered low-quality DNA and removed 3' adapter sequences using Trim
134 Galore. The obtained reads were cleaned using FastQC software
135 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and we then evaluated
136 the content and quality of the nucleotide bases within the sequencing data. Next, we
137 conducted a comparative analysis with the reference genome (*Eriocheir sinensis*;
138 NCBI Taxonomy ID: 95602). For each sample, sequence alignment with the reference
139 genome sequences was carried out using Tophat (Trapnell 2009).

140 *Transcriptome assembly*

141 Raw reads arising from the Illumina sequencing were pre-processed by
142 removing adaptor sequences, low-quality reads (reads with ambiguous base reads, or
143 'N'), and duplication sequences. Remaining sequences were then assembled using
144 SOAPdenovo software (BGI, Shenzhen, China) with default settings. Firstly, we used
145 Trim Galore to filter low-quality reads and remove 3' adapter sequences, and then
146 used FastQC software to clean reads and evaluate the performance of different k-mers.
147 Next, the clean reads were combined by de Bruijn graphs and SOAPdenovo software

148 based on sequence overlap to form longer fragments (without ambiguous 'N' reads),
149 to create contigs. Contigs were then connected using an undetermined bases, or 'N',
150 to represent the unknown sequence between each pair of contigs to form scaffolds.
151 Gaps between scaffolds can be filled by paired-end reads of sequencing to obtain
152 sequences with the least number of Ns and cannot be extended on either end. To
153 obtain unique gene sequences, we used TGI Clustering tools to cluster the transcripts.
154 Lastly, protein-coding sequences were predicted using Trinity software and translated
155 into amino acid sequences. The obtained transcripts were compared with the National
156 Center for Biotechnology Information (NCBI) non-redundant protein (Nr) database,
157 and UniProt using BLASTx (Basic Local Alignment Search Tool) searching with an
158 E-value<0.00001. Based on the results of Nr annotation, we use the Blast 2GO
159 software (<https://www.blast2go.com/>) to analyze functional annotation by gene
160 ontology terms (GO; <http://www.geneontology.org>). The transcripts were also aligned
161 to the Kyoto Encyclopedia of Genes and Genomes (KEGG) enKaryotic Orthologous
162 Group (KOG), a manually annotated and reviewed protein sequence database (Swiss
163 Prot) database to predict and classify functions to perform pathway annotation
164 searching with a similarity>30% and an E-value<0.00001, and then merged all
165 annotation information.

166 *Differential expression analysis*

167 To estimate the expression level (relative abundance) of a specific transcript
168 expressed as fragments per kilobase per million fragments mapped (FPKM) by using
169 RSEM software with default parameter settings (He et al.2013). The FPKM value for

each transcript was measured in reads per kilo base of transcript sequence per million mapped reads (Mortazavi 2008). The expression level of each transcript was transformed using $\log_2(\text{FPKM}+1)$. We used DESeq software to screen all differential expressed genes (DEGs) and calculate the relative change in transcript expression (Simon Anders, 2010). We used two-fold changes in expression, and p-values < 0.05 , as the threshold with which to judge the significance of differentiated gene expression.

Gene Ontology functional enrichment analysis for differentially expressed genes (DEGs)

We annotated the DEGs to analyze the potential consequential changes of function in *E. sinensis* following enrofloxacin treatment. In order to do this, we used GO terms in accordance with previously published procedures (Liu et al. 2011). This analysis firstly mapped all DEGs to GO terms in the database by calculating gene numbers for every term followed by an ultra-geometric test to find significantly enriched GO terms in DEGs compared to the transcriptome background. The formula was defined as follows:

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i} \binom{N-M}{n-i}}{\binom{N}{n}}$$

Where, N represents the number of all genes with GO annotation; n represents the number of DEGs in N; M represents the number of all genes annotated to specific GO terms; and m represents the number of DEGs in M. The calculated p-value was subjected to Bonferroni correction. A corrected p-value < 0.05 was defined as

191 'threshold'. GO terms were considered significantly enriched in the DEGs.

192 *Differentially Expressed Genes (DEGs) pathway analysis*

193 We used the BLASTall (<http://nebc.nox.ac.uk/bioinformatics/docs/blastall.html>)
194 program to annotate the pathways of differentially expressed genes (DEGs) against
195 the KEGG database. Enriched DEGs pathways were identified according to the same
196 formula as in the GO analysis. Here, N represented the number of all genes with
197 KEGG annotation, while n was the number of DEGs in N, M was the number of all
198 genes annotated to specific pathways, and m was the number of DEGs in M (Liu et al.
199 2011).

200 *qRT-PCR verification*

201 Quantitative RT-PCR (qRT-PCR) was used to verify the expression level of
202 DEGs that were identified by RNA-Seq analysis. Primers were designed using Primer
203 5 software and SpTub-b was used as a reference gene (Yan et al. 2006; West et al.
204 2010). Reactions were performed in a 25µl volume composed of 2µl cDNA, 0.5µl
205 forward primer and reverse primer (10µM), 12.5µl SYBR Premix Ex Taq (2X) and
206 9.5µL RNase-free H₂O. The thermal cycling program was 95°C for 30s, followed by
207 40 cycles of 95°C for 5s, 60°C for 30s, and 72°C for 30s. Melting curve analysis was
208 performed by the end of qRT-PCR to confirm PCR specificity.

209 **Results**

210 *Illumina sequencing and quality assessment*

211 In order to examine the effect of enrofloxacin upon the *E. sinensis*
212 transcriptome, we performed RNA-seq using the Illumina sequence platform. After
213 filtering and quality checks of the raw reads, there were 88,228,728 and 88,888,706
214 clean reads for the control (kongbai3) and treatment groups (shiyan4), respectively.
215 There were approximately 78 million (78,843,613) and 87 million (87,628,922)

216 trimmed reads with trim rates of 98.27% and 98.58% for kongbai3 and shiyan4
217 samples, respectively. Meanwhile, the respective average length of reads was
218 115.77bp and 118.18bp and GC percentages were 53.06% and 50.86%, respectively
219 (Table 1), indicating successful sequencing of the *E. sinensis* transcriptome. Trimmed
220 reads were then used for the subsequent analysis.

221 *Comparative analysis with the reference genome*

222 Trimmed reads from the *E. sinensis* transcriptome were compared with the
223 reference genome sequence. The total mapped rates of reads with the reference
224 genome were 65.70% in the control group (kongbai3) and 65.37% in the treatment
225 group (shiyan4). There were around 36 million (36,829,410) unique mapped reads for
226 the control group (kongbai3) and 45 million (45,028,754) for the treatment group
227 (shiyan4), accounting for 47.48 % and 52.07% of the total reads, respectively. There
228 was approximately 14 million multiple (14,135,167) mapped reads for the control
229 group (kongbai3) and 11 million (11,498,745) for the treatment group (shiyan4),
230 representing 18.22% and 13.30% of the total reads, respectively. Reads mapped in
231 proper pairs accounted for 43.05% in the control group (kongbai3) and 46.34% in the
232 treatment group (shiyan4) (Table 2).

233 Additionally, we compared protein sequences from the samples with common
234 data genes, and functional annotation was performed by analyzing similarity between
235 genes. Protein sequences were compared with KOG, GO, and KEGG databases.
236 Transcript annotation in Swissprot and TrEMBL accounted for 65.65% and 72.62%
237 of total transcripts (Table 3) (Figure 1). There were approximately 59.05%, 66.16%,
238 37.57%, of transcripts in KOG, GO, and KEGG, respectively (Table 3). We
239 determined transcripts by KOG classification. In total, there were 9,328 transcripts
240 clustered into 25 functional categories (Figure S1). The cluster of 'signal transduction

mechanisms', 'posttranslational modification, protein turnover, chaperones' and 'intracellular trafficking, secretion, and vesicular transport' occupied 25.93% of the total number of transcripts (2,419 transcripts, Figure S1). Using GO and KEGG database analysis transcripts, we found that most transcripts were enriched in cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems (Figure S2 and Figure S3).

Analysis of differential expression genes (DEGs)

To identify DEGs of *E. sinensis*, we used the Cuffdiff program to generate *E. sinensis* gene expression profiles (Figure 2 and Figure 3). This program identified 1,327 DEGs which were markedly up-regulated and 1,468 DEGs which were markedly down-regulated in the differentially expressed genes, which indicates that enrofloxacin affects *E. sinensis* gene expression.

Gene Ontology (GO) annotation of differentially expressed genes (DEGs)

We classified DEGs according to GO classification following enrofloxacin treatment in *E. sinensis* in order to investigate biological functions in which DEGs might be involved. GO is an international standardized gene function classification system which offers a dynamic-updated controlled vocabulary and a strictly defined concept to comprehensively describe the properties of genes and their products in any organism (Conesa 2008). Based upon homologous genes, we categorized 2,795 significant DEGs of *E. sinensis* into 3,785 GO terms consisting of three domains: biological processes, cellular components and molecular function (Figure 4 and Figure 5). It was clear that the dominant distributions referred to 'plasma membrane', 'cell periphery', 'extracellular region', 'plasma membrane part', 'membrane region' and 'plasma membrane region'. We also identified a high proportion of DEGs assigned to calcium ion binding, nutrient reservoir activity, structural constituent of

266 ribosome, ribosome, lipid transporter activity, iron ion binding, monooxygenase
267 activity, aromatase activity, and a few DEGs were assigned to the oxidation-reduction
268 process, translational initiation, membrane, cytoplasmic part and hydrolase activity
269 (Figure 4) (Table S1).

270 *Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differential*
271 *expressed genes (DEGs)*

272 We analyzed the biological pathways that were active in our samples in order to
273 investigate biological behavior. DEGs were mapped to the KEGG database and
274 enriched to important pathways, such as metabolism and signal transduction, based on
275 the whole transcriptome background. There were 2,795 significant DEGs mapped to
276 the reference canonical pathways using the Kyoto Encyclopedia of Genes and
277 Genomes (KEGG) (Kanehisa et al. 2008). These DEGs were assigned to 290 KEGG
278 pathways. Many DEGs were found in multiple pathways; however, many genes were
279 also restricted to a single pathway. These pathways included metabolism, genetic
280 information processing, cellular processes, organismal systems, and environmental
281 information processing (Figure 6). The most significantly enriched system was the
282 metabolic pathway (465 DEGs). The other significantly enriched pathway divided
283 into four categories including organismal systems (369 DEGs), environmental
284 information processing (216 DEGs), cellular processes (181 DEGs), and genetic
285 information processing (162 DEGs) (Figure 6) (Table S2). KEGG pathway analysis is
286 a useful tool for the prediction of potential genes and their functions at a whole
287 transcriptome level. These prediction pathways, together with GO and COG analysis,
288 will facilitate the annotation of transcripts and investigations of gene function in
289 future studies.

290 *Candidate genes involved in metabolism pathways*

291 Energy and material metabolism pathways included amino sugar and nucleotide
292 sugar metabolism (17 DEGs), steroid hormone biosynthesis (9 DEGs) (Morris 2007),
293 tyrosine metabolism (5 DEGs), glutathione metabolism (9 DEGs), arginine and
294 proline metabolism (10 DEGs), histidine metabolism (4 DEGs), citrate cycle (TCA
295 cycle) (7 DEGs), vitamin B6 metabolism (2 DEGs), beta-Alanine metabolism (6
296 DEGs), glycolysis / gluconeogenesis (8 DEGs), pentose phosphate pathway (3 DEGs),
297 pantothenate and CoA biosynthesis (2 DEGs), and oxidative phosphorylation (2
298 DEGs). Most of the DEGs involved in energy and material metabolism pathways
299 were substantially up-regulated, while all DEGs mapped to the steroid hormone
300 biosynthesis were down-regulated, which may have biological relevance for the drug
301 metabolism of *E. sinensis* after enrofloxacin stimulation.

302 Some DEGs enriched in the drug metabolic pathways included drug metabolism
303 - cytochrome P450 (7 DEGs), drug metabolism - other enzymes (11 DEGs), and
304 metabolism of xenobiotics by cytochrome P450 (7 DEGs), and degradation of
305 aromatic compounds (1 DEGs).

306 In the energy metabolism pathways, certain genes such as *XLOC_008932*,
307 *XLOC_000685* (Citrate cycle (TCA cycle)), *XLOC_003803*, *XLOC_025917*
308 (glycolysis / gluconeogenesis), *XLOC_023453*, *XLOC_013643* (pentose phosphate
309 pathway), *XLOC_014937* (oxidative phosphorylation) were up-regulated . While
310 others, such as *XLOC_003571* (citrate cycle (TCA cycle)) and *XLOC_010640*
311 (oxidative phosphorylation) were down-regulated following enrofloxacin treatment.

312 In the material metabolism pathways, *XLOC_013761*, *XLOC_017898*,
313 *XLOC_019355* (amino sugar and nucleotide sugar metabolism), *XLOC_022762*,
314 *XLOC_021523* (pantothenate and CoA biosynthesis), *XLOC_006723*, *XLOC_023156*,
315 *XLOC_020900*, *XLOC_016810*, *XLOC_001765* (glutathione metabolism) were

upregulated ,while others, such as *XLOC_009493*, *XLOC_010145*, *XLOC_002451*,
XLOC_010148 (steroid hormone biosynthesis), *XLOC_024909*, *XLOC_019159*,
XLOC_017926 (amino sugar and nucleotide sugar metabolism), *XLOC_007784*,
XLOC_017789, *XLOC_019705* (various types of N-glycan biosynthesis), were
down-regulated following enrofloxacin treatment . These findings were consistent
with the GO enrichment analysis and the concentration of enrofloxacin in the *E.*
sinensis hepatopancreas cells, mostly by influencing metabolism and trans-membrane
transport.

Candidate genes involved in signal transduction

In the present analysis, classes of genes that maintain relatively steady-state
levels of gene expression included those controlling tissue remodeling,
immunoregulation, cell-cycle progression, apoptosis and growth. High-throughput
sequencing efforts revealed that a large number of molecules were highly enriched in
signal pathways. Of these, we focused on key genes involved in the endocrine system
and other factor-regulated calcium reabsorption (8 DEGs), glutamatergic synapse (8
DEGs), TGF-beta signaling pathway (9 DEGs), cGMP - PKG signaling pathway (13
DEGs), HIF-1 signaling pathway (8 DEGs), MAPK signaling pathway (15 DEGs),
p53 signaling pathway (5 DEGs), and GABAergic synapse (3 DEGs).

Interestingly, the key components of the MAPK signaling pathway (Li et al 2013)
include MAP kinases, ERK1/2, and p38, which were identified in our data set, and
were all dramatically up-regulated in *E. sinensis*. MAPKs are composed of three
different major families, the extracellular signal regulated kinase (ERK) family, which
regulate different processes via a protease cascade. In this putative pathway, each
cascade is triggered by extracellular signals and result in the activation of MAPK
kinase (MAPKKK/MEKK), followed by activation of MAPK kinase

(MAPKK/MEK/MKK) and MAPK/ERK, finally leading to function in a diverse array of substrates and NF- κ B proteins (Leuthner et al. 2000; Harper et al. 2001; Takeda et al. 2002; Cho et al. 2003). However, since our knowledge of MAPK pathways in aquatic invertebrates is largely unclear, urgent research is required in order to fully clarify the role of this pathway, such as ERK, ERK1/2 which would for a valuable reference resource for crabs and other important crustaceans. In conclusion, a number of DEGs from the hepatopancreas of microbially-challenged *E. sinensis* were characterized to be associated with TGF- β , cGMP - PKG, HIF-1 and MAPK pathways.

Collectively, the results of our DEGs pathway analysis support the fact that the concentration of enrofloxacin in hepatopancreas cells mostly affected metabolism and transmembrane transport. Moreover, numerous genes in the hepatopancreas of microbially-challenged *E. sinensis* were characterized and associated with TGF- β , cGMP - PKG, HIF-1 and MAPK pathways.

Accuracy of the Illumina sequencing data, and the expression profile of the genes identified, were further confirmed by qRT-PCR and a cohort of these genes, such as *FKBP4*, *ND2*, *ATPeF1B*, *gltA*, *ATP5A1*, *cyt-b5*, *ERK*, were specifically identified. It is is very likely that further functional studies will identify these specific genes as new drug metabolism genes (Table 4).

Verification of the differential expression of differentially expressed genes

The primers of seven genes that were suggested to be related to drug metabolism as a result of significant differences after GO and KEGG analysis, and with clear functional implication, were designed to verify the expression of DEGs obtained from RNA-Seq analysis. All primer sequences are listed in Table 4. Data showed that the up-regulation or down-regulation of these seven genes were consistent with results

366 arising from RNA-Seq. Consequentially, these results indicated that qRT-PCR and
367 RNA-Seq results were reliable overall, but further studies are still required to confirm
368 verification (Figure 7).

369 Discussion

370 Illumina sequencing, a rapid and cost-effective method, provides an ideal means
371 with which to analyze *E. sinensis* transcriptomes. This method was successfully used
372 to study the transcriptome of the accessory sex gland and testis from the Chinese
373 mitten crab (He 2013), citrus red mite, and *Carcinus maenas* transcriptome
374 (Verbruggen 2015).

375 In this study, we found that enrofloxacin up-regulated 2,795 DEGs and
376 down-regulated 1,468 DEGs in *E. sinensis*. We categorized 1,327 *E. sinensis*
377 significant DEGs into 3,785 GO terms consisting of three domains: biological
378 processes, cellular components and molecular function. We identified a high
379 proportion of DEGs assigned to membrane fractions including the plasma membrane,
380 membrane region and plasma membrane region. A few DEGs assigned to the
381 transport protein pathway, and the activation of related enzymes, including calcium
382 ion binding, lipid transporter activity, iron ion binding, monooxygenase activity, and
383 aromatase activity. Studies show that a large number of genes are involved in the
384 oxidation-reduction process, translational initiation, membrane, cytoplasmic part and
385 hydrolase activity. This indicated that the metabolism of enrofloxacin may affect
386 multiple biological functions. In total, 2,795 DEGs were assigned to 290 KEGG
387 pathways. The significantly enriched pathway was divided into two categories
388 including metabolism and signal transduction pathways.

389 At present, the consensus of opinion is in aquatic animals, 98% of enrofloxacin
390 exists in the prototype form. The main metabolite of enrofloxacin is ciprofloxacin that

391 is form by the removal of the ethyl form of the enrofloxacin, but the content of less
392 than 2% in aquatic animals. (Interrel, L et al. 2000). By analyzing the molecular
393 structure of enrofloxacin, we found that an alkyl carbon atom attached to the nitrogen
394 atom has a hydrogen atom (α -hydrogen atom), which is oxidized to a α -hydroxyl
395 group. The formation of hydroxyl amine is an unstable intermediate and will
396 automatically divide. Drug metabolism enzymes CYP₄₅₀ play an important role in this
397 oxidation process. In our study, we found that the expression of some genes in the
398 CYP₄₅₀ enzyme system was up-regulated. We also found that *gshB* was upregulated
399 which in glutathione metabolish gene. It combines with aromatic compounds
400 containing halogen elements to form a combination that can dissolve in water and
401 directly excreted through urine or bile, that is conducive to reduce the concentration
402 of enrofloxacin in vivo. Therefore, we conclude that *gshB* and the CYP₄₅₀ enzyme
403 system plays a role in the metabolism of enrofloxacin in the hepatopancreas of *E.*
404 *sinensis*. We found that most of the DEGs were distributed in energy metabolism
405 processes, such as oxidative phosphorylation, phosphonate and phosphinate
406 metabolism, citrate cycle (TCA cycle), succinate metabolism, and oxaloacetate
407 metabolism. These metabolic processes not only produce energy, but also participate
408 in the trans-membrane transport of substance. This indicates that enrofloxacin is likely
409 to be transported by active transport. Some DEGs are distributed by the signaling
410 pathway and may play roles as drugs or target cells in drug receptors, thus
411 representing an important aspect of signaling regulation.

412 Most importantly, we identified and compared differences between genes related
413 to both drug metabolism and to signal transduction. Many potential candidate genes
414 related to gene regulation in *E. sinensis* were identified. Results indicated that drug
415 metabolism may be a complex biological process involving many changes in gene

416 expression, and most probably acts via transmembrane transport, hepatic processing,
417 energy biogenesis and protein synthesis. These findings greatly extend the existing
418 sequence resources relating to *E. sinensis* and provide abundant genetic information
419 with which to further understand the molecular mechanisms of drug metabolism.

420 **Date Archiving**

421 Sequencing reads are available in the NCBI SRA database (SRX1855752 and
422 SRX1940515).

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426 National Natural Resources Platform and the Shanghai University Knowledge Service
427 Platform.

428 **Supplementary data**

429 **Figure S1.** EnKaryotic orthologous group (KOG) classification.

430 **Figure S2.** Histogram of the enriched categories arising from the GO annotation of
431 transcripts in *E. sinensis*.

432 **Figure S3.** Histogram of the enriched KEGG pathways of transcripts in *E. sinensis*.

433 **Table S1.** GO analysis for the differential express genes of the *Eriocheir sinensis*.

434 **Table S2.** KEGG pathway analysis for the differential express genes of the *Eriocheir*
435 *sinensis*.

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531 **Table and Figure legends**

532 **Table 1.** Summary of reads arising from *E. sinensis* transcriptome sequencing.

533 **Table 2.** Statistical results of trimmed read mapping with a reference genome.

534 **Table 3.** Statistical results of gene functional annotation.

535 **Table 4.** Oligonucleotide primers for the qRT-PCR used to validate differential
536 expression genes (DEGs).

537 **Figure 1.** Venn diagram depicting database annotation.

538 **Figure 2.** Effect of enrofloxacin treatment upon the gene expression profile of *E.*
539 *sinensis* following enrofloxacin treatment.

540 **Figure 3.** Scatter plot of differential expression genes cluster in the expression profile
541 of *E. sinensis*.

542 **Figure 4.** Histogram of the enriched category arising from the GO annotation of
543 differential expression genes (DEGs) in *E. sinensis* following enrofloxacin treatment.

544 **Figure 5.** Scatter plot of the enriched GO annotation of differential expression genes
545 genes (DEGs) in *E. sinensis* following enrofloxacin treatment.

546 **Figure 6.** Scatter plot of the enriched pathway of KEGG annotation of differential
547 expression genes (DEGs) in *E. sinensis* following enrofloxacin treatment.

548 **Figure 7.** Comparison of the expression levels of seven genes (*FKBP4*, *ND2*,
549 *ATPeF1B*, *gltA*, *ATP5A1*, *Cyt-b5*, *ERK*) acquired from RNA-Seq and qRT-PCR.

550

551 **Table 1. Summary of reads arising from *E. sinensis* transcriptome sequencing.**

Sample	Raw reads	Trimmed reads	Average length	Trim rate	GC rate
Kongbai3	80,228,728	78,843,613	115.77bp	98.27%	53.06%
Shiyan4	88,888,706	87,628,922	118.18bp	98.58%	50.86%

552 **Table 2. Statistical results of trimmed read mapping with a reference genome.**

Map to genome	Kongbai3		Shiyan4	
	Reads numbers	percentage	Reads numbers	percentage
Total reads	77,573,418	100.00%	86,473,988	100.00%
Total mapped	50,964,577	65.70%	56,527,499	65.37%
Uniquely mapped	36,829,410	47.48%	45,028,754	52.07%
Multiple mapped	14,135,167	18.22%	11,498,745	13.30%
Reads1 mapped	18,392,163	23.71%	22,462,610	25.98%
Reads2 mapped	18,437,247	23.77%	22,566,144	26.10%
Mapped to '+'	18,403,492	23.72%	22,506,437	26.03%
Mapped to '-'	18,425,918	23.75%	22,522,317	26.05%
Non - splice reads	28,656,320	36.94%	33,729,657	39.01%
Splice reads	8,173,090	10.54%	11,299,097	13.07%
Reads mapped in proper pairs	33,399,100	43.05%	40,075,716	46.34%

553 **Table 3. Statistical results of gene functional annotation.**

Database	Number of transcripts	Percentage(%)
Annotation in COD	9,670	61.21%
Annotation in KOG	9,328	59.05%
Annotation in NR	11,510	72.86%
Annotation in NT	2,628	16.64%
Annotation in PFAM	9,018	57.09%
Annotation in Swissprot	10,370	65.65%
Annotation in TrEMBL	11,472	72.62%
Annotation in GO	10,452	66.16%
Annotation in KEGG	5,935	37.57%
Annotation in at least one database	11,975	75.81%
Annotation in all database	1,635	10.35%
Total transcripts	15,797	100%

554

555 **Table 4. Oligonucleotide primers for the qRT-PCR used to validate differential**
 556 **expression genes (DEGs).**
 557

Gene name	Predict function	GO category	Pathway name	Primer name	Nucleotide sequence (5'-3')	Expected product
ACTIN	-	-	-	ACTIN-F	TCGTGCGAGACATCAAGGAA	177bp
				ACTIN-R	A GGAAGGAAGGCTGGAAGAG TG	
FKBP4	hypothetical	protein complex	-	FKBP4-F	TGCGACAGTTGGTGAGGAGT	114bp
	protein DAPPUDRAF T_204206	localization (Biological process) Nucleolus(Cellular component)		FKBP4-R	AAGCAAAGCAGAAGGCAGA GG	
ATPeF1B	F1F0-ATP synthase beta subunit	small molecule metabolic process(Biological process)	Oxidative phosphorylation	ATPeF1B-F	CGGGAGATGGAGTCAAGAGG	185bp
				ATPeF1B-R	AT CGTATCACCACCACCAAGAA GG	
ND2	NADH dehydrogenase subunit 2	energy derivation by oxidation of organic compounds(Biological process)	Oxidative phosphorylation	ND2-F	CGCCACCACTACCTCTTATTT	208bp
				ND2-R	C TAGCACAGATCCTAATGCCT GA	
gltA	putative citrate synthase	oxidation-reduction process, carboxylic acid metabolic process(Biological process)	Citrate cycle(TCA CYCLE)	gltA-F	CCAGTTCTCAGCAGCCATCA	170bp
				gltA-R	C CACGGTAAAGGTTGCGGTAG AT	
ATP5A1	mitochondrial ATP synthase subunit alpha precursor	small molecule metabolic process (Biological process)\	Oxidative phosphorylation	ATP5A1-F	TTGGCGATGGTGAGGAT	140bp
				ATP5A1-R	GAGGAGCAGGTAGCCGTCAT	
Cyt-b5	hypothetical	galactose catabolic process (Biological process)	-	Cyt-b5-F	CCACAATCAGCGACCACACC	239bp
	protein DAPPUDRAF T_303198	Lysosome(Cellular component)		Cyt-b5-R	GTCCAACCATCCAGCCTTA GG	
ERK	mitogen-activated protein kinase	oxoacid metabolic process, organic acid metabolic process(Biological process)	VEGF signaling pathway	ERK-251F	TGATTGAAGGAGGACCGTGG	251bp
				ERK-251R	TA TGTGAGAGCAGGAGTGGTAG AG	

558

559 **Figure 1. Venn diagram depicting database annotation.**

560 **Figure 2. Effect of enrofloxacin treatment upon the gene expression profile of *E.***
561 ***sinensis* following enrofloxacin treatment.**
562 Volcanic plot of the degree of differences in the expression profile of *E. sinensis* after
563 treatment with enrofloxacin. X-axis, $\log_2(\text{fold change})$; Y-axis, $-\log_2(\text{Pvalue})$. Gray,
564 differential expression genes; black, not differential expression genes. Each dot
565 represents one gene.

566 **Figure 3. Scatter plot of differential expression genes cluster in the expression**
567 **profile of *E. sinensis*.**

568 A broken line in the figure represents a gene's expression in different samples. The
569 graph shows that all the genes under each cluster are similar in all samples.

570 **Figure 4. Histogram of the enriched category arising from the GO annotation of differential expression genes (DEGs) in *E. sinensis***
571 **following enrofloxacin treatment.**
572 GO terms (X-axis) were grouped into three main ontologies: biological process, cellular component, and molecular function. The Y-axis
573 indicates the number of DEGs.

574 **Figure 5. Scatter plot of the enriched GO annotation of differential expression**
575 **genes (DEGs) in *E. sinensis* following enrofloxacin treatment.**
576 Scatter plot of the degree of differences in the expression profile of *E. sinensis*. X-axis,
577 Rich factor; Y-axis, pathway name. A corrected p-value < 0.05 was defined as
578 ‘threshold’. GO terms were considered significantly enriched in the DEGs. The size
579 of the dots indicates the number of DEGs contained in each term.

581 **Figure 6. Scatter plot of the enriched pathway of KEGG annotation of**
582 **differential expression genes genes (DEGs) in *E. sinensis* following enrofloxacin**
583 **treatment.**
584 Scatter plot of the degree of differences in the expression profile of *E. sinensis*. X-axis,
585 Rich factor; Y-axis, pathway name. A corrected p-value < 0.05 was defined as
586 ‘threshold’. KEGG pathway were considered significantly enriched in the DEGs. The
587 size of the dots to indicate the number of DEGs contained in each pathway.

588 **Figure 7. Comparison of the expression levels of seven genes (*FKBP4*, *ND2*,**

589 *ATPeF1B*, *gltA*, *ATP5A1*, *Cyt-b5*, *ERK*) acquired from RNA-Seq and qRT-PCR.

590 Negative values represent the gene expression of *E. sinensis* following oral gavage

591 enrofloxacin treatment which was down-regulated while positive values represent

592 up-regulated levels of gene expression.

Supplementary data**Figure S1. EnKaryotic orthologous group (KOG) classification.**

[S] Function unknown, [Z] Cytoskeleton, [Y] Nuclear structure, [W] Extracellular structures, [V] Defense mechanisms, [U] Intracellular trafficking, secretion, and vesicular transport, [T] Signal transduction mechanisms, [R] General function prediction only, [Q] Secondary metabolites biosynthesis, transport and catabolism, [P] Inorganic ion transport and metabolism, [O] Posttranslational modification, protein turnover, chaperones, [N] Cell motility, [M] Cell wall/membrane/envelope biogenesis, [L] Replication, recombination and repair, [K] Transcription, [J] Translation, ribosomal structure and biogenesis, [I] Lipid transport and metabolism, [H] Coenzyme transport and metabolism, [G] Carbohydrate transport and metabolism, [F] Nucleotide transport and metabolism, [E] Amino acid transport and metabolism, [D] Cell cycle control, cell division, chromosome partitioning, [C] Energy production and conversion, [B] Chromatin structure and dynamics, [A] RNA processing and modification.

Figure S2. Histogram of the enriched categories arising from the GO annotation of transcripts in *E. sinensis*.

categories (x-axis) were grouped into three main ontologies: biological process, cellular component, and molecular function. The y-axis indicates the statistical the percent of genes (%).

Figure S3. Histogram of the enriched KEGG pathways of transcripts in *E. sinensis*.

X-axis, KEGG pathway categories; y-axis, statistical significance of enrichment.

Table S1. GO analysis for the differential express genes of the *Eriocheir sinensis*.

GO_ID	Term	Type	All_annotated _num	All_num_this _term	DEGs_this _term	UP	Down	Expected	Pvalue	FDR
GO:0005886	plasma membrane	cellular_component	6715	814	275	158	117	204.15	1.20E-09	8.46E-06
GO:0071944	cell periphery	cellular_component	6715	857	286	164	122	214.93	2.30E-09	8.46E-06
GO:0005576	extracellular region	cellular_component	6715	447	160	91	69	112.11	1.00E-07	0.000245267
GO:0044459	plasma membrane part	cellular_component	6715	332	124	67	57	83.27	2.30E-07	0.000423085
GO:0098589	membrane region	cellular_component	6715	218	85	44	41	54.67	2.70E-06	0.00397332
GO:0005509	calcium ion binding	molecular_function	6715	211	84	44	40	54.97	5.80E-06	0.007112733
GO:0098590	plasma membrane region	cellular_component	6715	172	69	34	35	43.14	7.70E-06	0.0080938
GO:0045735	nutrient reservoir activity	molecular_function	6715	8	8	7	1	2.08	2.10E-05	0.018803778
GO:0016324	apical plasma membrane	cellular_component	6715	57	29	12	17	14.3	2.30E-05	0.018803778
GO:0003735	structural constituent of ribosome	molecular_function	6715	111	48	2	46	28.92	5.40E-05	0.0397332
GO:0016020	membrane	cellular_component	6715	2387	662	368	294	598.66	6.60E-05	0.044148
GO:0005840	ribosome	cellular_component	6715	142	56	6	50	35.61	9.60E-05	0.058864
GO:0005319	lipid transporter activity	molecular_function	6715	47	24	12	12	12.24	0.0002	0.105114286

GO:0005905	coated pit	cellular_component	6715	35	19	10	9	8.78	0.0002	0.105114286
GO:0044425	membrane part	cellular_component	6715	1776	499	274	225	445.42	0.00028	0.137349333
GO:0045177	apical part of cell	cellular_component	6715	85	36	15	21	21.32	0.00034	0.1563575
GO:0016028	rhabdomere	cellular_component	6715	10	8	1	7	2.51	0.00042	0.166523158
GO:0005506	iron ion binding	molecular_function	6715	101	42	18	24	26.31	0.00043	0.166523158
GO:0098805	whole membrane	cellular_component	6715	520	163	79	84	130.42	0.00043	0.166523158
GO:0004497	monooxygenase activity	molecular_function	6715	63	29	5	24	16.41	0.00046	0.169234
GO:0042995	cell projection	cellular_component	6715	279	94	48	46	69.97	0.0006	0.210228571
GO:0005929	cilium	cellular_component	6715	52	24	17	7	13.04	0.00075	0.250840909
GO:0035150	regulation of tube size	biological_process	6715	27	15	10	5	6.9	0.00084	0.268726957
GO:0016712	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxyge...	molecular_function	6715	22	13	1	12	5.73	0.00104	0.299575714
GO:0070330	aromatase activity	molecular_function	6715	22	13	1	12	5.73	0.00104	0.299575714
GO:0007155	cell adhesion	biological_process	6715	228	79	46	33	58.26	0.00113	0.299575714
GO:0022610	biological adhesion	biological_process	6715	228	79	46	33	58.26	0.00113	0.299575714

GO:0016705	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxyge...	molecular_function	6715	102	41	15	26	26.57	0.00114	0.299575714
GO:0005615	extracellular space	cellular_component	6715	122	46	29	17	30.6	0.00123	0.309036
GO:0022626	cytosolic ribosome	cellular_component	6715	18	11	2	9	4.51	0.00126	0.309036
GO:0007628	adult walking behavior	biological_process	6715	7	6	4	2	1.79	0.00151	0.334982632
GO:0090659	walking behavior	biological_process	6715	7	6	4	2	1.79	0.00151	0.334982632
GO:0005198	structural molecule activity	molecular_function	6715	226	79	22	57	58.87	0.00154	0.334982632
GO:0008509	anion transmembrane transporter activity	molecular_function	6715	85	35	19	16	22.14	0.00156	0.334982632
GO:0022892	substrate-specific transporter activity	molecular_function	6715	379	124	65	59	98.73	0.0016	0.334982632
GO:0015849	organic acid transport	biological_process	6715	45	21	11	10	11.5	0.00171	0.334982632
GO:0046942	carboxylic acid transport	biological_process	6715	45	21	11	10	11.5	0.00171	0.334982632
GO:0005537	mannose binding	molecular_function	6715	9	7	3	4	2.34	0.00173	0.334982632
GO:0045202	synapse	cellular_component	6715	134	49	22	27	33.61	0.00187	0.352806667

GO:0044421	extracellular region part	cellular_component	6715	188	65	44	21	47.15	0.00199	0.3660605
GO:0098862	cluster of actin-based cell projections	cellular_component	6715	24	13	9	4	6.02	0.00212	0.380163333
GO:1902652	secondary alcohol metabolic process	biological_process	6715	40	19	7	12	10.22	0.00217	0.380163333
GO:0004806	triglyceride lipase activity	molecular_function	6715	16	10	2	8	4.17	0.00227	0.382950455
GO:0016125	sterol metabolic process	biological_process	6715	43	20	8	12	10.99	0.00229	0.382950455
GO:0044699	single-organism process	biological_process	6715	3925	1044	585	459	1002.93	0.00274	0.448020444
GO:0009925	basal plasma membrane	cellular_component	6715	12	8	3	5	3.01	0.00282	0.451077391
GO:0044456	synapse part	cellular_component	6715	91	35	17	18	22.82	0.00309	0.476737083
GO:0007424	open tracheal system development	biological_process	6715	77	31	15	16	19.68	0.00311	0.476737083
GO:0015291	secondary active transmembrane transporter activity	molecular_function	6715	89	35	16	19	23.18	0.00392	0.554014118
GO:0008061	chitin binding	molecular_function	6715	17	10	7	3	4.43	0.00424	0.554014118

Table S2. KEGG pathway analysis for the differential express genes of the *Eriocheir sinensis*.

KO_ID	Term	Type	All_annotated_num	All_num_this_term	DEGs_this_term	UP	Down	Pvalue	FDR
ko03010	Ribosome	Genetic Information Processing	2316	91	45	1	44	8.84E-08	2.56E-05
ko00520	Amino sugar and nucleotide sugar metabolism	Metabolism	2316	35	17	12	5	0.0013897 65	0.2015159 25
ko04974	Protein digestion and absorption	Organismal Systems	2316	22	11	7	4	0.0075042 75	0.5131187 5
ko00140	Steroid hormone biosynthesis	Metabolism	2316	17	9	0	9	0.0097444 02	0.5131187 5
ko00051	Fructose and mannose metabolism	Metabolism	2316	20	10	4	6	0.0107276 4	0.5131187 5
ko05133	Pertussis	Human Diseases	2316	15	8	8	0	0.0139883 4	0.5131187 5
ko00254	Aflatoxin biosynthesis	Metabolism	2316	3	3	0	3	0.014155	0.5131187 5
ko00521	Streptomycin biosynthesis	Metabolism	2316	3	3	1	2	0.014155	0.5131187 5

ko04973	Carbohydrate digestion and absorption	Organismal Systems	2316	10	6	2	4	0.0166597	0.5368125 56
ko05204	Chemical carcinogenesis	Human Diseases	2316	25	11	1	10	0.0230802 9	0.6239653 18
ko00603	Glycosphingolipid biosynthesis - globo series	Metabolism	2316	8	5	5	0	0.0236676 5	0.6239653 18
ko05206	MicroRNAs in cancer	Human Diseases	2316	48	18	9	9	0.0265098 4	0.6402572 55
ko00053	Ascorbate and aldarate metabolism	Metabolism	2316	11	6	3	3	0.0292675 6	0.6402572 55
ko04514	Cell adhesion molecules (CAMs)	Environmental Information Processing	2316	14	7	4	3	0.0320974 7	0.6402572 55
ko04911	Insulin secretion	Organismal Systems	2316	20	9	4	5	0.0332300 6	0.6402572 55
ko04261	Adrenergic signaling in cardiomyocytes	Organismal Systems	2316	37	14	9	5	0.0441526 6	0.6402572 55
ko04971	Gastric acid secretion	Organismal Systems	2316	21	9	6	3	0.0460493 9	0.6402572 55
ko00604	Glycosphingolipid biosynthesis - ganglio series	Metabolism	2316	4	3	3	0	0.0463755 2	0.6402572 55

ko00950	Isoquinoline alkaloid biosynthesis	Metabolism	2316	4	3	1	2	0.0463755	0.6402572
								2	55
ko04744	Phototransduction	Organismal Systems	2316	4	3	1	2	0.0463755	0.6402572
								2	55
ko00270	Cysteine and methionine metabolism	Metabolism	2316	18	8	7	1	0.0474549	0.6402572
								7	55
ko04142	Lysosome	Cellular Processes	2316	72	24	15	9	0.0485712	0.6402572
								4	55
ko00965	Betalain biosynthesis	Metabolism	2316	2	2	0	2	0.0585951	0.7183481
								1	25
ko00500	Starch and sucrose metabolism	Metabolism	2316	22	9	2	7	0.0617767	0.7183481
								5	25
ko04961	Endocrine and other factor-regulated calcium reabsorption	Organismal Systems	2316	19	8	4	4	0.0651647	0.7183481
								9	25
ko00982	Drug metabolism - cytochrome P450	Metabolism	2316	16	7	2	5	0.0679937	0.7183481
								2	25
ko05217	Basal cell carcinoma	Human Diseases	2316	10	5	2	3	0.0689505	0.7183481
								7	25
ko00983	Drug metabolism - other enzymes	Metabolism	2316	29	11	6	5	0.0693577	0.7183481
								5	25

ko04020	Calcium signaling pathway	Environmental Information Processing	2316	33	12	7	5	0.0796201 8	0.7273838
ko04916	Melanogenesis	Organismal Systems	2316	23	9	3	6	0.0805442	0.7273838
ko00380	Tryptophan metabolism	Metabolism	2316	20	8	6	2	0.0864546 1	0.7273838
ko04713	Circadian entrainment	Organismal Systems	2316	20	8	4	4	0.0864546 1	0.7273838
ko04310	Wnt signaling pathway	Environmental Information Processing	2316	44	15	8	7	0.0892894 5	0.7273838
ko00980	Metabolism of xenobiotics by cytochrome P450	Metabolism	2316	17	7	1	6	0.0923475 5	0.7273838
ko00061	Fatty acid biosynthesis	Metabolism	2316	5	3	0	3	0.0952637 4	0.7273838
ko04740	Olfactory transduction	Organismal Systems	2316	5	3	3	0	0.0952637 4	0.7273838
ko00514	Other types of O-glycan biosynthesis	Metabolism	2316	14	6	3	3	0.0978205 8	0.7273838
ko01040	Biosynthesis of unsaturated fatty acids	Metabolism	2316	14	6	3	3	0.0978205 8	0.7273838

ko04080	Neuroactive ligand-receptor interaction	Environmental Information Processing	2316	14	6	4	2	0.0978205 8	0.7273838
ko00350	Tyrosine metabolism	Metabolism	2316	11	5	3	2	0.1020197	0.7396428 25
ko04972	Pancreatic secretion	Organismal Systems	2316	31	11	5	6	0.1061895	0.7510964 63
ko04970	Salivary secretion	Organismal Systems	2316	21	8	5	3	0.1113194	0.7657370 91
ko04520	Adherens junction	Cellular Processes	2316	28	10	7	3	0.1161808	0.7657370 91
ko04670	Leukocyte transendothelial migration	Organismal Systems	2316	28	10	7	3	0.1161808	0.7657370 91
ko00830	Retinol metabolism	Metabolism	2316	18	7	2	5	0.1209156	0.7792338 67
ko04350	TGF-beta signaling pathway	Environmental Information Processing	2316	25	9	3	6	0.1272741	0.7853082 77
ko04512	ECM-receptor interaction	Environmental Information Processing	2316	25	9	8	1	0.1272741	0.7853082 77

ko00052	Galactose metabolism	Metabolism	2316	15	6	3	3	0.1310723	0.7918951 46
ko00627	Aminobenzoate degradation	Metabolism	2316	3	2	2	0	0.1474753	0.8618098 6

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